Production of SO_2 Binding Compounds and SO_2 by *Saccharomyces* during Alcoholic Fermentation and the Impact on Malolactic Fermentation

A. Wells, J.P. Osborne*

Department of Food Science and Technology, Oregon State University, Corvallis, OR, 97331, USA

Submitted for publication: May 2011
Accepted for publication: August 2011

Key words: SO_2, *Saccharomyces*, *Oenococcus oeni*, Malolactic Fermentation, Acetaldehyde, Pyruvic Acid

The objective of this study was to investigate the production of SO_2 and SO_2 binding compounds by wine yeast and the impact of the production of these compounds on the MLF at various time points during alcoholic fermentation. Fermentations were observed for a number of commercial wine yeasts in a synthetic grape juice and Pinot gris juice and SO_2, acetaldehyde, pyruvic acid and α-ketoglutarate. Measurements were taken at multiple time points during the fermentation. Samples were taken from the fermentations at weekly intervals, sterile filtered, and inoculated with *O. oeni* strain VFO to induce MLF. Significant differences between the amount of SO_2, acetaldehyde and pyruvic acid produced by the various yeast strains were noted. Some yeast strains such as FX10, CK S102, F15 and M69, produced significantly higher SO_2 concentrations than other yeast strains and MLF was inhibited in these wines. Insignificant free SO_2 was measured, indicating that bound SO_2 rather than free SO_2 was responsible for inhibition. At almost all time points of the alcoholic fermentation, acetaldehyde bound SO_2 was determined to be the dominant species of bound SO_2 present, suggesting that MLF inhibition by bound SO_2 was due to acetaldehyde bound SO_2.

INTRODUCTION

Malolactic fermentation (MLF) is a common practice in winemaking, particularly red winemaking (Lafon-Lafourcade et al., 1983; Wibowo et al., 1985) where certain lactic acid bacteria (LAB) decarboxylate L-malic acid to L(+)-lactic acid and carbon dioxide (CO2). This results in a reduction in wine acidity but also may contribute desirable flavours, aromas, and mouthfeel (Kunkee *et al.*, 1965; Kunkee, 1967; Lafon-Lafourcade *et al.*, 1983; Fleet *et al.*, 1984; Wibowo *et al.*, 1988; Van Vuurren & Dicks, 1993). Induction of the MLF can be problematic due to the effects of low pH (Davis *et al.*, 1985; Wibowo *et al.*, 1985), temperature (Britz & Tracey, 1990), and/or antagonistic interactions between wine yeast (*Saccharomyces*) and malolactic bacteria (*Oenococcus*) (Beelman *et al.*, 1982; King & Beelman, 1986; Lonvaud-Funel *et al.*, 1988; Wibowo *et al.*, 1988; Henick-Kling & Park, 1994; Osborne & Edwards, 2006; 2007). Some researchers have proposed that inhibition of MLF is due to the removal of nutrients by the faster growing *Saccharomyces* (Beelman *et al.*, 1982; Kunkee, 1967). However, recent studies have demonstrated that the removal of nutrients by yeast does not always explain the observed inhibition of *O. oeni* (Patynowski *et al.*, 2002; Larsen *et al.*, 2003; Osborne and Edwards 2007). Instead, it is suggested that yeast may produce metabolites toxic to the malolactic bacteria (Dick *et al.* 1992; Capucho & San Ramao, 1994; Osborne & Edwards, 2006) including ethanol (Costello *et al.*, 1983; Davis *et al.*, 1985), medium chain fatty acids (Edwards & Beelman 1987; Versari *et al.*, 1999), antibacterial proteins/peptides (Dick *et al.*, 1992; Osborne & Edwards, 2007; Mendoza *et al.*, 2010; Nehme *et al.*, 2010) and SO_2 (King & Beelman, 1986; Henick-Kling & Park, 1994; Eglinton & Henschke, 1996; Larsen *et al.*, 2003). Of these compounds, SO_2 is most commonly implicated (Henick-Kling & Park, 1994; Larsen *et al.*, 2003; Osborne & Edwards, 2006) as it is a known antimicrobial against malolactic bacteria (Carr *et al.*, 1976).

SO_2 is produced by *Saccharomyces* as an intermediate during the assimilatory reduction of sulfate to sulfite (Avram & Bakalinsky, 1997; Thomas & Surdin-Kerjan, 1997; Donalies & Stahl, 2002). In an aqueous system, SO_2 exists in equilibrium between molecular SO_2, bisulfite ions, and sulfite anions in a pH dependant manner. In addition, the bisulfite ion can also exist in a free or bound form (Fugelsang & Edwards, 2007). In wine, the bisulfite ion will react with carbonyl compounds such as acetaldehyde, forming adducts such as hydroxysulfonic acids. Although acetaldehyde

*Corresponding author: E-mail: james.osborne@oregonstate.edu Tel.: 541 737 6494; Fax: 541 737 1877

binds most strongly with SO_2, other carbonyl compounds found in wine, such as pyruvic acid and α-ketoglutaric acid, may also bind with SO_2 (Fornachon, 1963; Rankine & Pocock, 1969; Burroughs & Sparks, 1973a; Azevedo *et al.*, 2007). While bound SO_2 is thought to have much weaker antimicrobial properties than free SO_2 (and in particular the molecular portion) (Eschenbruch, 1974; Hammond & Carr, 1976; Hinze & Holzer, 1986; Rose & Pilkington, 1989), a number of researchers have suggested that bound SO_2 may be more antimicrobial than previously believed, particularly towards bacteria (Hood, 1983; Larsen *et al.*, 2003; Osborne & Edwards, 2006). For example, while Larsen *et al.* (2003) and Osborne & Edwards (2006) found a strong correlation between yeast production of SO_2 and the inhibition of *O. oeni,* they reported that free SO_2 was not detected and that the SO_2 measured was present in unidentified bound forms.

Wine yeast is known to produce different amounts of the major SO_2 binding compounds acetaldehyde (Romano *et al.*, 1994), α-ketoglutarate (Rankine, 1968) and pyruvic acid (Rankine, 1965). Therefore, fermentations with different yeast strains may result in wines containing various concentrations of acetaldehyde, pyruvic acid and α-ketoglutarate bound SO_2. However, the impact of these different forms of bound SO_2 on the MLF is unknown. If some forms of bound SO_2 are more inhibitory to malolactic bacteria than others, then this may explain the varied ability of wine yeast to inhibit the MLF despite the production of similar amounts of SO_2 (Larsen *et al.,* 2003; Osborne & Edwards, 2006). It may also explain why bacterial inhibition is strongest early in the alcoholic fermentation, but decreases near the end or after completion of the fermentation (Larsen *et al.,* 2003) as shifts in the concentrations of SO_2 binding compounds could be expected to occur as the fermentation proceeds.

Improved success in the induction of MLF requires a better understanding of the compatibility between different strains of yeast and malolactic bacteria. If different yeast strains produce different amounts of SO_2 binding compounds, this may have to be accounted for when considering yeast/ bacterial combinations. In addition, the role bound SO_2 plays in causing problematic MLFs may need to be considered. Knowledge regarding the production of SO_2 binding compounds by yeast may also minimize SO_2 use through the choice of yeast strain. For example, use of yeast strains that produce low amounts of binding compounds may allow the winemaker to add less SO_2 to maintain an effective level for the antimicrobial purposes. This reduction of SO_2 use is beneficial, as there is mounting consumer resistance to the excessive use of SO_2 and other chemical preservatives in wine (Du Toit & Pretorious, 2000). Therefore the objective of this study was to investigate the production of SO_2 and SO_2 binding compounds by wine yeast and the impact the production of these compounds has on the MLF at various time points during the alcoholic fermentation.

MATERIALS AND METHODS

Microorganisms

Active-dry forms of *S. cerevisiae* obtained were strains MERIT.ferm, RUBY.ferm (Chr. Hansen, Hørsholm, Denmark), CK S102 (Springer Oenologie, France), Zymaflore FX10, Zymaflore F15, (Laffort, France), Lalvin BM45, Lalvin V1116 (K1), Lalvin M69, and *S. bayanus* Uvaferm 43 (Lallemand, Montréal, Canada). Yeast was maintained on potato dextrose agar (PDA) (Difco, Franklin Lakes, NJ, USA) slants stored at 4°C. The strain *Oenococcus oeni* used in this study was the freeze-dried form of DSM 7008, Viniflora oenos (Chr. Hansen).

Starter culture preparation

Yeast was transferred from PDA slants to 250mL yeast peptone dextrose (YPD) broth (10g/L yeast extract, 20g/L peptone, 20g/L dextrose, pH 7.0) and incubated aerobically at 25°C for 48 h. Cells were harvested by centrifugation (4,000 x *g* for 20 minutes) and suspended in 0.2M phosphate buffer (27.80g/L $NaH_2PO_4 \cdot H_2O$, 28.38g/L Na_2HPO_4, pH 7.0) before inoculation. To initiate malolactic fermentation, freeze-dried bacteria were rehydrated with 0.2M phosphate buffer for 15 min before inoculation.

Enumeration

Microbial viabilities were determined using serial tenfold dilutions (0.1% peptone) and using appropriate media. Yeast cells were grown on YPD agar while bacteria were enumerated using media based on de Man, Rogosa and Sharpe (MRS) agar (20g/L Tryptone, 5g/L peptone, 5g/L yeast extract, 5g/L glucose, 200mL apple juice, 1mL Tween 80 [5%, w/w, solution], 20 g/L agar, pH 4.5). Plates were incubated aerobically at 25°C for 48 h (yeast) or 7 days (bacteria) prior to counting.

Synthetic Grape Juice

A synthetic grape juice (pH 3.5, 24 °Brix) based on Wang *et al.* (2003) and modified as per Osborne & Edwards (2006) was utilized. Basic juice parameters were pH 3.50, titratable acidity (TA) 6.5g/L, 24.0 °Brix, and yeast assimilable nitrogen (YAN) 250mg/L. YAN was calculated as the sum of the concentrations of ammonia and the molar proportion of the α-amino nitrogen present in amino acids except proline.

Pinot Gris Juice

Pinot gris grapes harvested from Oregon State University's Woodhall Vineyard (2008) were pressed and the juice was stored at -20°C until required. No SO_2 was added to the juice following pressing. Basic juice parameters (pH, TA, °Brix) were measured using standard methods, while YAN was calculated as the sum of α-amino nitrogen measured by NOPA (Dukes and Butzke, 1998) combined with ammonia measured by enzymatic test kit (R-Biopharm, Darmstadt, Germany). After thawing, juice parameters were pH 3.35, TA 6.8 g/L, 24.5 °Brix, and 150 mg/L YAN.

Alcoholic Fermentation

Both the synthetic grape juice and Pinot gris grape juice were sterile filtered (0.45μm PES disposable Ultripor® filters, PALL Corp. East Hills, NY, USA) into sterile 4L glass carboys. The final volume of juice in each fermentor was 3L. Alcoholic fermentations were induced by the addition of yeast at a rate of $1x10^6$ CFU/mL. Yeast strains MERIT. ferm, RUBY.ferm, CK S102, Lalvin M69, Zymaflore FX10, Zymaflore F15, Lalvin BM45 and Uvaferm 43 were used

for fermentations performed in synthetic grape juice while yeast strains V1116, FX10, BM45 and M69 were used for fermentations performed in Pinot gris juice. V1116, FX10, BM45 and M69 were chosen for the Pinot gris fermentations as these strains had demonstrated high production of either SO_2 or SO_2 binding compounds in previous trials. All fermentations were performed in triplicate at 21°C. Aseptic sampling during the fermentation was accomplished using a nitrogen siphon system. Completion of the alcoholic fermentation (< 2g/L reducing sugars) was determined by CliniTest®.

Fermentation Monitoring

During alcoholic fermentation, 150mL samples were aseptically removed and 100mL was sterile filtered through 0.45µm disposable Nalgene PES membrane filter unit (NalgeNuno International, Rochester, NY, USA) into sterilized milk dilution bottles. Freeze-dried *O. oeni* VFO were rehydrated in 0.2M phosphate buffer for 10 minutes before inoculation into the 100mL sterile filtered samples at initial populations of approximately $1x10^6$CFU/mL. Bottles were incubated at 25°C with bacterial viable cell populations and L-malic acid (enzymatic test kit, R-Biopharm) being measured weekly. The remaining sample (≈50 mL) was used to analyze yeast viable cell populations and free/bound SO_2 by the aeration-oxidation method (Buechsenstein and Ough, 1978). Samples were also analyzed for acetaldehyde, pyruvic acid (enzymatic test kit, R-Biopharm) and α-ketoglutarate (enzymatic assay as described by Peynaud *et al.* (1966)).

Estimating Metabolite-bound SO_2

The concentration of the individual bound SO_2 species was estimated using an equation formulated by Burroughs and Sparks (1973a, b):

$[x] = [X] * [S] / K + [S]$

Where [x] = molecular concentration of bisulfite bound carbonyl; [X] = total molecular concentration of the carbonyl compound; [S] = molecular concentration of free SO_2; and K = dissociation constant for the specific binding compound (constants from Burroughs and Sparks, 1973a). By analyzing the acetaldehyde, pyruvic acid, α-ketoglutarate and free SO_2 content of the wines the concentrations of each carbonyl bound by SO_2 can be calculated. This equation has been used in a number of studies to estimate the concentration of bound SO_2 species (Rankine, 1965; 1967; 1968; Burroughs and Sparks, 1973a, b; Barbe *et al.,,* 2000; Lea *et al.* 2000).

Statistics

Statistical analysis was accomplished using SAS version 9.1 (SAS Institute Inc, Cary, NC, USA) with Tukey's HSD test for mean separation.

RESULTS

After inoculation into synthetic grape juice, all yeast strains achieved populations of $1x10^8$ CFU/mL or greater within 10 days (Fig. 1) except for CK S102, which reached a peak population of 7×10^7 CFU/mL after 14 days. Viable cell counts for all yeast declined slowly after this point reaching a minimum of around $1x10^5$ CFU/mL by day 49. All fermentations were completed in 28 days (< 2g/L reducing sugars as assessed by CliniTest®). Yeast growth during fermentation of Pinot gris juice showed a similar trend with populations peaking after 7 days of fermentation (Fig. 2). However, the decline of yeast viable cells was less than what was seen in the synthetic grape juice fermentations, aside from yeast strain M69 which declined to below 1×10^4 CFU/mL after 49 days. In Pinot gris juice all fermentations were completed by day 35.

Yeast strains produced significantly different amounts of SO_2, acetaldehyde, and pyruvic acid during alcoholic fermentation in both the synthetic grape juice and Pinot gris juice (Figs 3 and 4, Table 1). The concentration of free SO_2 measured during the fermentations was low ranging from 2.1 (MERIT.ferm) to 9.0 mg/L (BM45) in synthetic grape juice (Table 1), and 2.1 (M69) to 3.3 mg/L (FX10 and BM45) in Pinot gris juice (Table 2). However, much higher levels of bound SO_2 were present in fermentations of both the synthetic and Pinot gris juice. For the synthetic grape juice, maximum concentrations of bound SO_2 ranged from 27.7 mg/L in fermentations performed by RUBY.ferm to 60.3mg/L in fermentations performed by M69 (Table 1). Maximum concentrations of SO_2 generally occurred between day 8 and day 15 of the fermentation (Fig. 3), coinciding with maximum yeast populations. A reduction in the amount of bound SO_2 occurred after this peak for most yeast strains although fermentations conducted by strains RUBY.ferm, BM45 and CK S102 displayed little reduction in bound SO_2 (Fig. 3).

Production of the major SO_2 binding compounds was also measured during the course of the fermentations as shown in Fig. 3. During fermentation in synthetic grape juice, acetaldehyde production was variable between yeast strains, peaking in most cases at the beginning of fermentation during the yeast exponential growth phase. After this, acetaldehyde concentrations decreased as the fermentation proceeded. Yeast strain Uvaferm 43 produced the highest amount of acetaldehyde followed by CK S102, while strain FX10 produced the lowest concentration of acetaldehyde as indicated in Table 1. For pyruvic acid, results showed a similar trend to acetaldehyde production with the maximum amount of pyruvic acid being produced early in the fermentation during exponential growth by the yeast. The only exception was yeast strain F15. This yeast produced a maximum concentration of pyruvic acid much later in the fermentation (day 28) compared to the other strains (Fig. 3F). Yeast strain Uvaferm 43 (Fig. 3H) produced the highest concentration of pyruvic acid reaching a maximum production of 223.5 mg/L pyruvic acid after two days of fermentation. Pyruvic acid concentrations tended to decrease less during the remainder of the fermentation compared to acetaldehyde concentrations. In particular, in fermentations conducted by RUBY.ferm, MERIT.ferm and M69, pyruvic acid concentrations remained high and decreased insignificantly after peaking during day 2 or day 8 of the fermentation (Fig. 3A, 3B, 3C). Concentrations of α-ketoglutarate produced by each yeast strain followed a similar trend to that of acetaldehyde and pyruvic acid production (data not shown). However, no significant differences were noted between the maximum concentrations of α-ketoglutarate produced by the various yeast strains (Table 1).

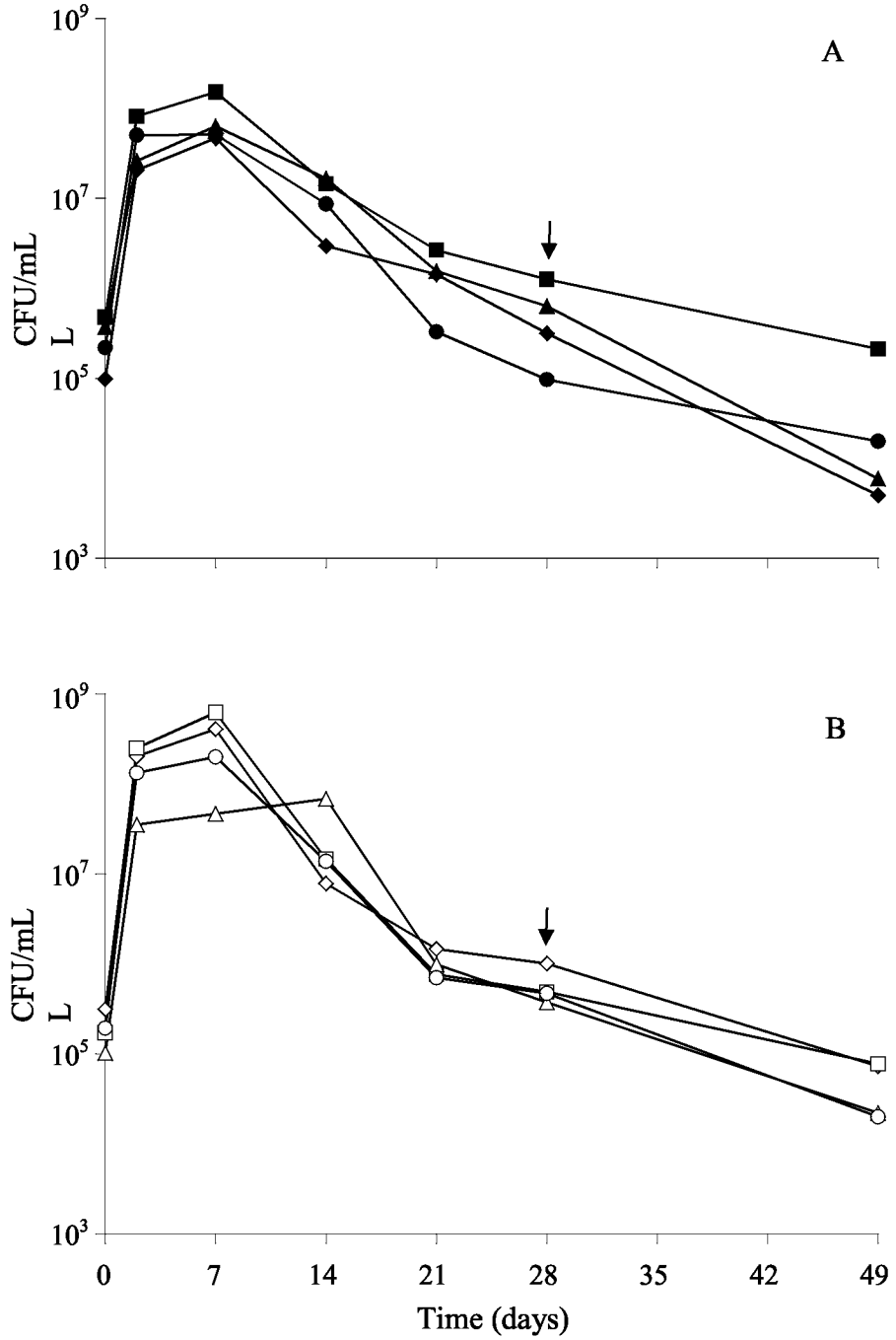

FIGURE 1
Growth of (A) *S. cerevisiae* strain RUBY.ferm (♦), MERIT.ferm (■), M69 (▲), FX10 (●), and (B) *S. cerevisiae* strain BM45 (◊), F15 (□), CK S102 (Δ), and *S. bayanus* Uvaferm 43 (○) during alcoholic fermentation in synthetic grape juice. Arrows indicate completion of alcoholic fermentation. Values are means from triplicate fermentations.

Production of SO_2 and SO_2 binding compounds during fermentation in Pinot gris juice yeast strains demonstrated similar trends to those observed during fermentation in synthetic grape juice (Fig. 4). Maximum concentrations of bound SO_2 ranged from 33.1 mg/L (M69) to 47.7 mg/L (FX10) as indicated in Table 2 and occurred between day 8 and day 15 of the fermentation (Fig. 4). Compared to fermentations in synthetic grape juice, M69 produced lower concentrations of bound SO_2 in Pinot gris fermentations (33.1 mg/L in Pinot gris versus 60.3 mg/L in synthetic grape juice), while FX10 and BM45 produced similar concentrations. Yeast production of SO_2 binding compounds during fermentation of Pinot gris juice also closely matched the trends observed in the synthetic grape juice. The highest

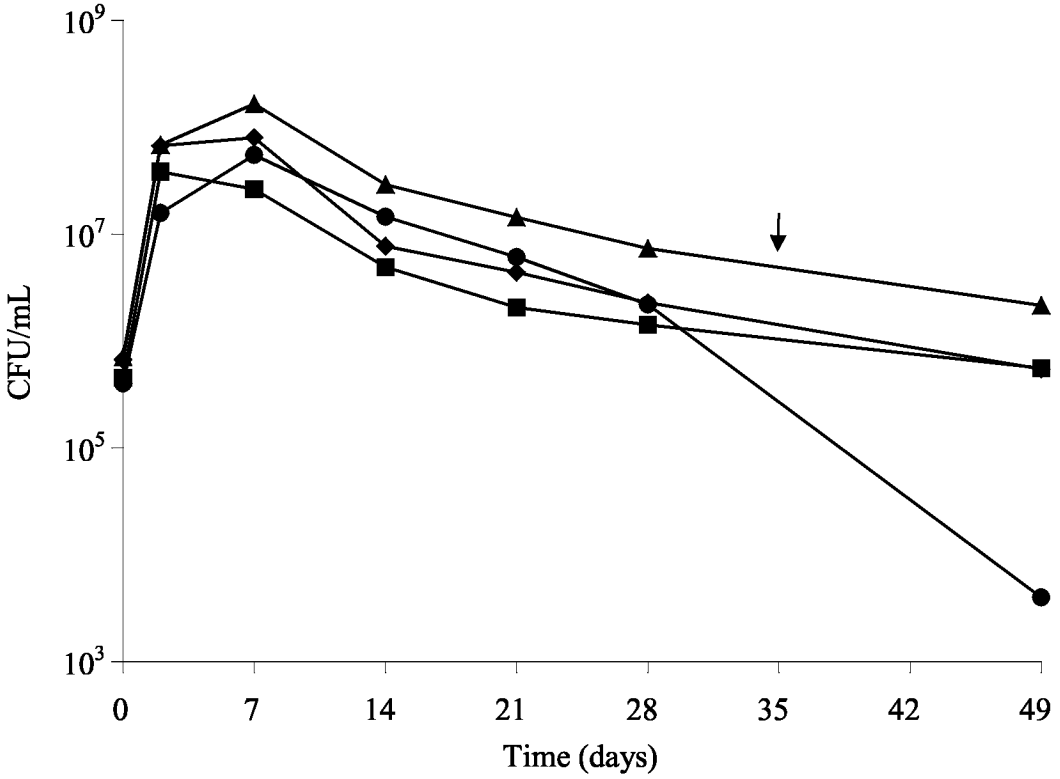

FIGURE 2
Growth of *S. cerevisiae* strain V1116 (♦), FX10 (■), BM45 (▲), and M69 (●) during alcoholic fermentation in Pinot Gris grape juice. Arrow indicates completion of alcoholic fermentation. Values are means from triplicate fermentations.

concentrations of acetaldehyde were produced at the beginning of the alcoholic fermentation with strain M69 producing a maximum of 105.1 mg/L (Fig. 4D, Table 2) by day 8. In addition, yeast strains produced higher amounts of acetaldehyde during fermentation in Pinot gris juice than they had during fermentation in synthetic media (Table 1 and 2). For example, FX10 produced a maximum of 35.0 mg/L of acetaldehyde during fermentation of synthetic grape juice and a maximum of 74.9 mg/L during fermentation of the Pinot gris juice.

Pyruvic acid results were similar to the synthetic grape juice fermentations in that the concentration of total pyruvic acid increased rapidly during the early part of fermentation. Unlike in the synthetic grape juice fermentations, pyruvic acid decreased as the alcoholic fermentation progressed with fermentations induced by M69, reaching a minimum of 5.0 mg/L on day 29 (Fig. 4D). In contrast to what was observed for acetaldehyde, all yeast strains produced lower concentrations of pyruvic acid during fermentation in Pinot gris juice than they did in synthetic grape juice (Table 2). Concentrations of α-ketoglutarate produced by each yeast strain followed a similar pattern to that of acetaldehyde and pyruvic acid production with maximum production occurring on either day 2 or day 8 of the alcoholic fermentation (data not shown). However, no statistically significant differences between yeast strains were noted for α-ketoglutarate production. Overall, α-ketoglutarate concentrations were higher in the Pinot gris fermentations than in the synthetic grape juice fermentations.

Concentrations of acetaldehyde bound SO_2, pyruvic acid bound SO_2 and α-ketoglutarate bound SO_2 were estimated using an equation formulated by Burroughs and Sparks (1973a, b). Acetaldehyde was present in higher concentrations than SO_2 (on a molar basis) at almost every sampling time for the majority of the yeast strains. Acetaldehyde so strongly binds SO_2 that in practice, not until nearly all the acetaldehyde is bound to SO_2, will pyruvic acid or α-ketoglutarate begin to be bound (Burroughs and Sparks, 1973a,b; Wurdig, 1989). This resulted in acetaldehyde bound SO_2 being the dominant and in most cases the only form of bound SO_2 present in the wines at any time during the fermentation. There were times when there were higher concentrations of SO_2 than acetaldehyde present and where the bound SO_2 was present partly as pyruvic acid bound SO_2. For example, in fermentations involving FX10 there were higher concentrations of SO_2 than acetaldehyde on day 7, 14, 28 and 49 of the fermentation (Fig. 3). For strain CK S102 there was 12.1 mg/L pyruvic acid bound on day 49 and 11.1 mg/L of pyruvic acid bound SO_2 present on day 8 of alcoholic fermentation with strain M69.

The impact of the production of SO_2 and SO_2 binding compounds during the alcoholic fermentation on *O. oeni* and

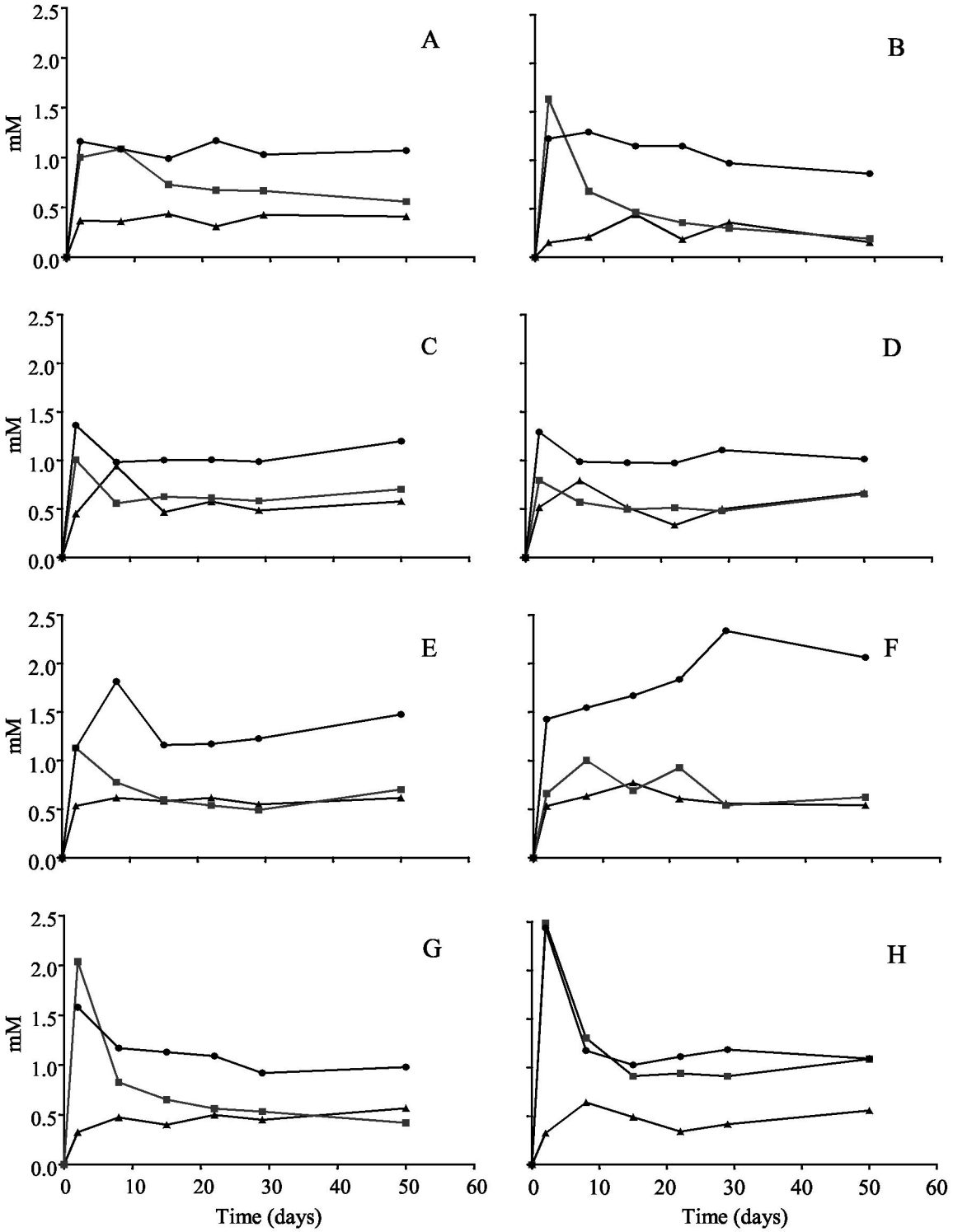

FIGURE 3
Concentration of acetaldehyde (■), pyruvic acid (●), and bound SO_2 (▲) during fermentation of synthetic grape juice by *S. cerevisiae* strain RUBY.ferm (A), MERIT.ferm (B), M69 (C), FX10 (D), BM45 (E), F15 (F), CK S102 (G), and *S. bayanus* Uvaferm 43 (H). Values are means from triplicate fermentations.

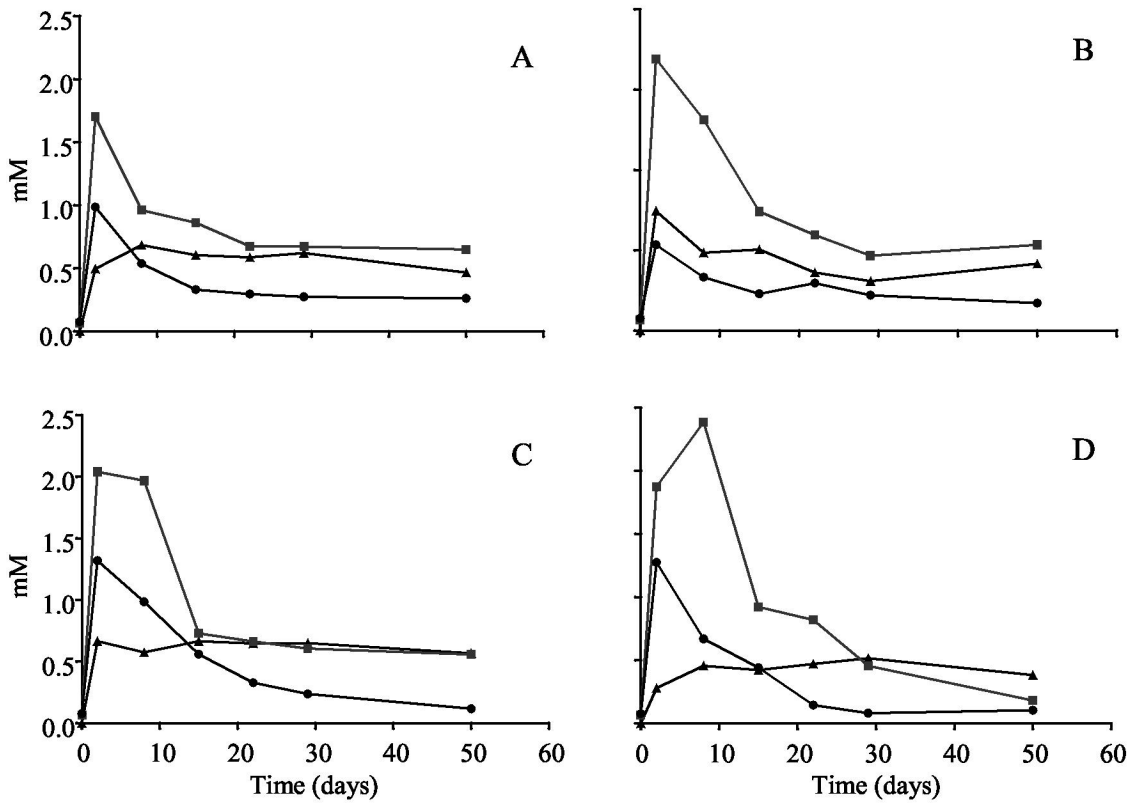

FIGURE 4
Concentration of acetaldehyde (■), pyruvic acid (●), and bound SO_2 (▲) during fermentation of Pinot Gris by *S. cerevisiae* strain V1116 (A), FX10 (B), BM45 (C), and M69 (D). Values are means from triplicate fermentations.

TABLE 1
Maximum concentrations of free and bound SO_2, acetaldehyde, pyruvic acid, and α-ketoglutarate produced by different *Saccharomyces* strains during alcoholic fermentation in synthetic grape juice. All values are in mg/L and are means of triplicate fermentations.

	Free SO_2	Bound SO_2	Acetaldehyde	Pyruvic acid	α-ketoglutarate
FX10	5.8^{ab}	50.7^{a}	35.0^{d}	113.8^{ab}	39.1^{a}
BM45	9.0^{a}	39.5^{bd}	49.8^{cd}	136.1^{ab}	42.9^{a}
M69	5.2^{ab}	60.3^{a}	44.4^{cd}	119.9^{ab}	37.7^{a}
CK S102	3.7^{b}	36.3^{b}	89.8^{ab}	113.7^{ab}	41.1^{a}
MERIT.ferm	2.1^{b}	28.3^{bc}	71.9^{bcd}	113.7^{ab}	37.2^{a}
F15	5.8^{ab}	49.4^{a}	44.2^{cd}	175.8^{abc}	47.1^{a}
Uvaferm 43	3.5^{b}	41.1^{abd}	109.7^{a}	223.5^{c}	31.5^{a}
RUBY.ferm	3.7^{b}	27.7^{bc}	47.9^{cd}	103.6^{b}	32.2^{a}

$^{a-d}$Values with no common superscripts are significantly different (Tukey's Studentized Range Test $p<0.05$)

TABLE 2
Maximum concentrations of free and bound SO_2, acetaldehyde, pyruvic acid, and α-ketoglutarate produced by different *Saccharomyces cerevisiae* strains during alcoholic fermentation in Pinot Gris juice. All values are in mg/L and are means of triplicate fermentations.

	Free SO_2	Bound SO_2	Acetaldehyde	Pyruvic acid	α-ketoglutarate
V1116	2.7^{a}	43.8^{a}	74.9^{b}	86.9^{a}	62.6^{a}
FX10	3.3^{a}	47.7^{a}	74.5^{b}	47.1^{b}	48.8^{a}
BM45	3.3^{a}	42.6^{a}	89.8^{ab}	86.7^{a}	57.2^{a}
M69	2.1^{a}	33.1^{b}	105.1^{a}	68.6^{a}	45.7^{a}

$^{a-d}$Values with no common superscripts are significantly different (Tukey's Studentized Range Test $p<0.05$)

the MLF was investigated by taking sterile filtering samples during the alcoholic fermentation, inoculating them with *O. oeni* VFO and following bacterial viable cells and malic acid degradation. Prior to yeast growth in synthetic grape juice (day 0) *O. oeni* VFO grew well (data not shown) and MLF was completed (malic acid < 0.2 g/L) between 7 and 14 days after inoculation (Fig. 5). Once yeast growth had begun, *O. oeni* VFO was impacted differently depending on which yeast strain was instigating the alcoholic fermentation. In fermentations conducted by using MERIT.ferm (Fig. 5B) and Uvaferm 43 (Fig. 5H) *O. oeni* VFO completed the MLF in 28 days or less at every stage of the alcoholic fermentation. In contrast, in samples fermented by CK S102, *O. oeni* VFO grew poorly and MLF was not completed at any of the sampling points during the alcoholic fermentation aside from day 0 (Fig. 5G). For yeast strain BM45, MLF was only completed in samples fermented for 2 days (Fig. 5E), while for strains RUBY.ferm (Fig. 5A) and M69 (Fig. 5C), MLF was completed in samples fermented for 2 and 7 days and for F15 MLF was completed in samples fermented for 2, 7 and 14 days (Fig. 5F). MLF was inhibited in all samples fermented by FX10 (Fig. 5D) except for the sample taken on day 49.

Similar impacts on the MLF were observed during alcoholic fermentation of the Pinot gris juice. *O. oeni* VFO grew well (data not shown) and MLF was completed in musts fermented by M69 (Fig. 6D) and FX10 (Fig. 6B), but was inhibited in samples fermented for 14, 21, 28 and 50 days by V1116 (Fig. 6A) and BM45 (Fig. 6C). Interestingly, while yeast strain M69 inhibited the MLF during fermentation of the synthetic grape juice it did not inhibit the MLF during fermentation in the Pinot gris juice. FX10 was also less inhibitory to the MLF during fermentation of Pinot gris, while BM45 showed the same inhibition of MLF in both synthetic and Pinot gris grape juice.

DISCUSSION

During alcoholic fermentation in synthetic grape juice and Pinot gris grape juice, strains of *S. cerevisiae* produced different amounts of total SO_2 with the differences being accounted for almost entirely by bound SO_2 as very little free SO_2 was measured during the alcoholic fermentation. Yeasts are known to produce a wide range of SO_2 concentrations during alcoholic fermentation (Rankine & Pocock, 1969) depending primarily on yeast strain, fermentation temperature and juice composition (Weeks, 1969; Eschenbruch, 1974; Henick-Kling & Park, 1994; Jarvis & Lea, 2000; Osborne & Edwards, 2006). In this study, the juice composition (either synthetic grape juice or Pinot gris juice) and the fermentation temperature were kept constant and so the differences in SO_2 production were likely due to variability between the yeast strains in their ability to produce SO_2. Differences noted between SO_2 production during alcoholic fermentation in the synthetic grape juice compared to Pinot gris juice may have been due to the difference in YAN concentrations. Osborne & Edwards (2006) reported that yeast produced higher concentrations of SO_2 under higher YAN conditions and in study the synthetic grape juice contained 250 mg/LYAN while the Pinot gris juice contained only 150 mg/L.

Aside from SO_2, yeasts also differ in their production of the major SO_2 binding compounds. While differences in yeast production of acetaldehyde have previously been reported (Weeks, 1969; Margalith, 1981; Martinez *et al.*, 1997) the production of pyruvic acid and α-ketoglutarate by wine yeast during alcoholic fermentation is less well documented (Rankine, 1965; 1967; 1968). In addition, this study reports on the production of all three major SO_2 binding compounds during the course of alcoholic fermentation as well as SO_2 production rather than final concentrations of these compounds in finished wines as reported by Rankine (1965; 1967; 1968). For acetaldehyde, maximum production occurred during the yeast exponential growth phase in agreement with previous reports (Amerine & Ough, 1964; Weeks, 1969; Margalith, 1981; Martinez *et al.*, 1997) and this is to be expected as acetaldehyde is an intermediate in the production of ethanol by the yeast. Although other researchers have proposed that the presence of SO_2 can induce the production of acetaldehyde (Weeks, 1969; Stratford *et al.*, 1987; Pilkington & Rose, 1988), this was not always observed in this study. For example, during fermentation in Pinot gris juice yeast strain M69 produced the lowest amount of SO_2 but the highest concentration of acetaldehyde. SO_2 induced production of acetaldehyde has been suggested to be a mechanism that yeast uses to tolerate higher SO_2 concentrations (Pilkington & Rose, 1988; Stratford *et al.*, 1987). This response was not apparent in this study and additional research on this topic is needed to determine the link between SO_2 and the production of acetaldehyde by *Saccharomyces*.

Differences in yeast production of pyruvic acid were noted while no statistical differences were found for α-ketoglutarate production. Yeast strains F15 and Uvaferm 43 produced higher concentrations of pyruvic acid than the other yeast strains in synthetic grape juice while in Pinot gris fermentations V1116, BM45 and M69 produced higher concentrations of pyruvic acid than FX10. Concentrations produced were in agreement with the findings of Rankine (1967; 1968). Maximum production of pyruvic acid and α-ketoglutarate generally occurred during the yeast exponential growth phase as expected, as these compounds are intrinsically involved in yeast metabolism and growth. Interestingly, higher concentrations of acetaldehyde, pyruvic acid and α-ketoglutarate were measured during fermentation in the synthetic grape juice than the Pinot gris juice. This may have been due to differences in pH and YAN concentrations between the two juices, as these factors are known to impact the production of acetaldehyde, pyruvic acid and α-ketoglutarate (Rankine, 1965; 1967; Rankine & Pocock, 1969).

While yeast produced different amounts of SO_2 and SO_2 binding compounds, they also influenced the MLF differently. These findings are consistent with those of other researchers who reported that yeast strains vary in their antagonism of malolactic bacteria (Larsen *et al.*, 2003; Osborne & Edwards, 2006). In the synthetic grape juice, fermentations FX10 and CK S102 exhibited the earliest inhibition of the MLF, occurring after 2 days growth, while RUBY.ferm and M69 inhibited MLF after 14 days growth and F15 only inhibiting MLF after 21 days growth. Conversely, the MERIT.ferm and Uvaferm 43 strains did not inhibit the MLF at any stage of the

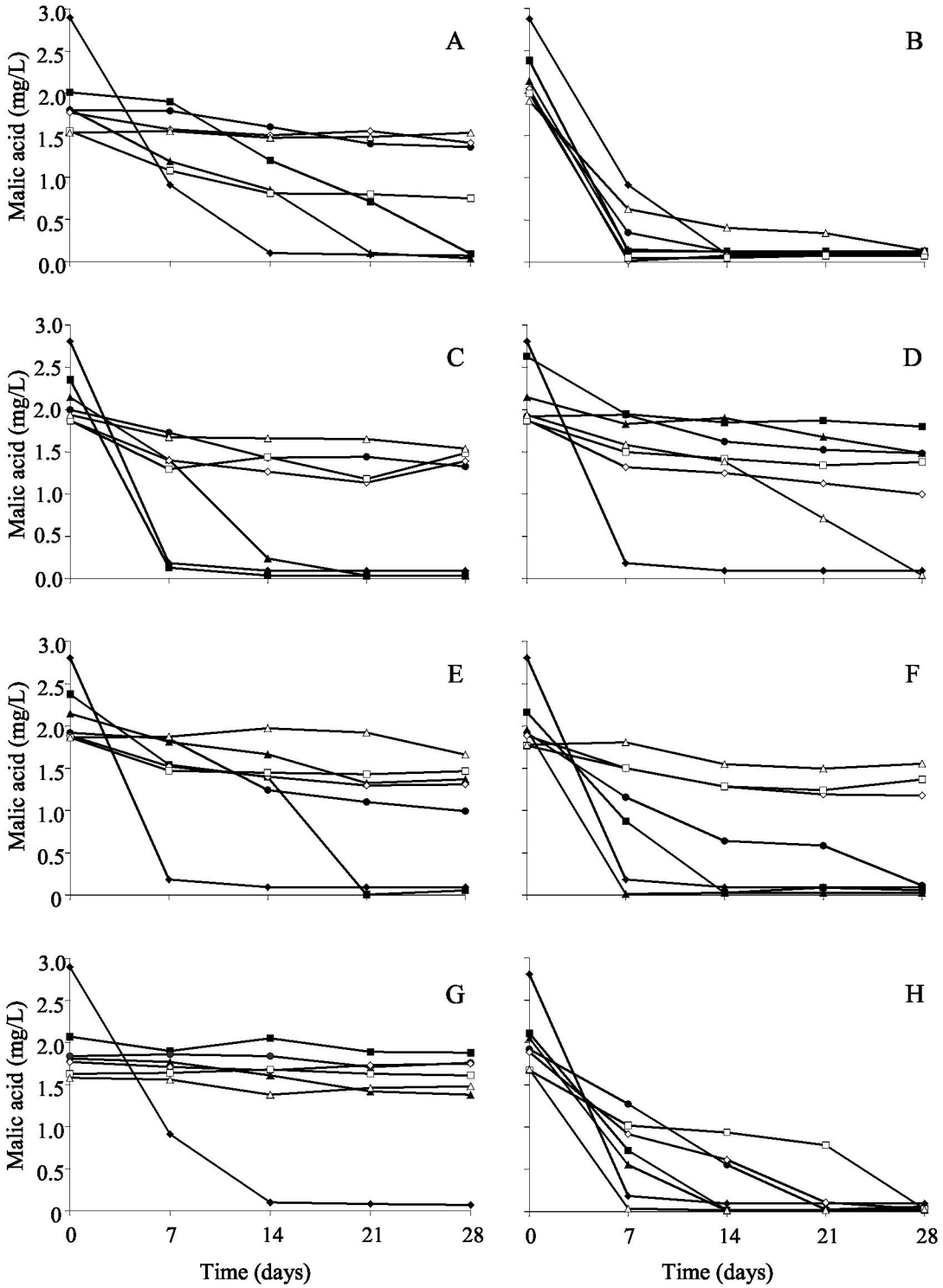

FIGURE 5

Degradation of malic acid in synthetic grape juice fermented by *S. cerevisiae* strain RUBY.ferm (A), MERIT.ferm (B), M69 (C), FX10 (D), BM45 (E), F15 (F), CK S102 (G), and *S. bayanus* Uvaferm 43 (H) for 0 (♦), 2 (■), 7 (▲), 14 (●), 21 (◊), 28 (□) and 49 (Δ) days. Values are means from triplicate fermentations.

alcoholic fermentation. Compared to synthetic grape juice fermentations, fermentations in Pinot gris were less inhibitory to MLF. For example FX10 inhibited MLF after 21 days growth in Pinot gris versus 2 days growth in synthetic grape juice. Inhibition of MLF was generally strongest during the early to mid stages of the alcoholic fermentation correlating with Larsen *et al.* (2003), who saw strong inhibition during the mid-alcoholic fermentation. Except in the case of FX10, relief of inhibition was not observed even after 49 days. It has been proposed that yeast lees contact can help stimulate

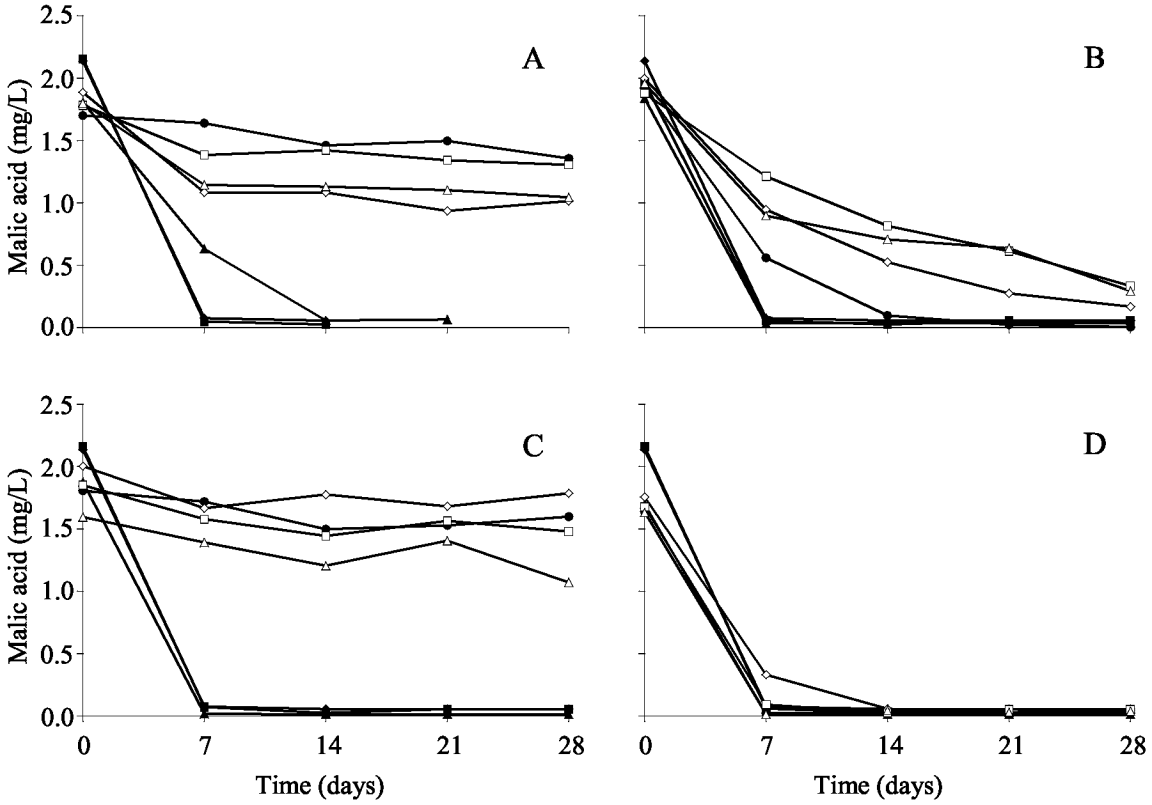

FIGURE 6
Degradation of malic acid in Pinot Gris juice fermented by *S. cerevisiae* strain V1116 (A), FX10 (B), BM45 (C), or M69 (D) for 0 (♦), 2 (■), 7 (▲), 14 (●), 21 (◊), 28 (□) and 49 (Δ) days. Values are means from triplicate fermentations.

the MLF by either replenishment of nutrients due to yeast autolysis (Beelman *et al.*, 1982; Patynowski *et al.*, 2002) or degradation or adsorption of toxic compounds responsible for the inhibition (Patynowski *et al.*, 2002). However, this was not observed in this study.

Inconsistency in the ability of a yeast strain to inhibit the MLF has often been attributed to the production of antibacterial compounds most commonly assosiated with SO_2 (Fornachon, 1968; Henick-Kling & Park, 1994; Larsen *et al.*, 2003). In this study, yeast strains (M69, FX10, F15) that produced high concentrations of SO_2 inhibited the MLF, while MERIT.ferm didn't and produced lower SO_2. Furthermore, M69 inhibited MLF only during fermentation in synthetic grape juice when high SO_2 concentrations were produced, but not during fermentation in Pinot gris juice when lower SO_2 concentrations were measured. However, yeast produced SO_2 could not always explain the observed MLF inhibition. For example, during growth in synthetic grape juice, yeast strains RUBY.ferm and MERIT. ferm produced low concentrations of SO_2 and yet RUBY. ferm inhibited the MLF after 14, 21, 28 and 49 days of fermentation, while MERIT.ferm did not inhibit MLF at any point of the alcoholic fermentation. Instead, the inhibition of MLF by RUBY.ferm is most likely caused by the production of an antibacterial peptide by this yeast strain as previously reported by Osborne and Edwards (2007). In addition, Nehme *et al.* (2010) and Mendoza *et al.* (2010) recently documented the production of an antibacterial peptide by yeast that can inhibit *O. oeni*.

Other possible inhibitory mechanisms include ethanol toxicity (Britz & Tracey 1990; Capucho & San Ramao 1994) and production of medium chain fatty acids (Edwards & Beelman 1987; Edwards *et al.*, 1990; Capucho & San Ramao 1994). It is unlikely that ethanol toxicity was primarily responsible for MLF inhibition given that some yeast did not inhibit *O. oeni* at any point during the alcoholic fermentation. For example, MERIT.ferm did not inhibit the MLF even at the end of fermentation when ethanol levels would have been at a maximum, while CK S102 inhibited MLF after only 2 days fermentation when ethanol levels relatively low. However, it is possible that increasing ethanol concentrations during the fermentation contributed to the MLF inhibition. For example, inhibition of MLF by yeast strain F15 increased as fermentation proceeded with maximum inhibition occurring from day 21 onwards. The production of medium chain fatty acids by yeast strains was not monitored in this study and so this mechanism cannot be discounted. However, the relative toxicity of medium chain fatty acids, the concentrations required to inhibit MLF, and whether yeasts are capable of producing these concentrations, has not been conclusively

determined (Edwards *et al.*, 1989, 1990; Cloete *et al.*, 2001).

In this study, inhibition of MLF by yeast produced SO_2 was most likely caused by bound SO_2 given that very low concentrations of free SO_2 were measured during the alcoholic fermentations. This is further evidence that bound SO_2 has an inhibitory action against *O. oeni* VFO as suggested by others (Fornachon, 1963; Hood 1983; Larsen *et al.*, 2003; Osborne & Edwards, 2006). However, what has not been established is whether the form of bound SO_2 present is important and whether different forms of bound SO_2 are more or less toxic to *O. oeni*. If different concentrations of acetaldehyde, pyruvic acid and α-ketoglutarate bound SO_2 are present during the alcoholic fermentation, then this may account for the varied ability of different yeast strains to inhibit the MLF. In this study, the concentrations of the major bound SO_2 species were calculated at multiple times during the alcoholic fermentation. Acetaldehyde bound SO_2 accounted for the majority of the bound SO_2 present during the fermentations and for almost every yeast strain and time point during the fermentation there was a higher concentration of acetaldehyde present than SO_2 (on a molar basis). Day 8 of the alcoholic fermentation performed by FX10 was one of the few sampling times where there was higher SO_2 levels than acetaldehyde, resulting in a large portion of the bound SO_2 being present as pyruvic acid bound SO_2. However, strong inhibition of MLF was still observed demonstrating that, in this case, a shift in the form of some of the bound SO_2 from acetaldehyde bound to pyruvic acid bound did not impact the inhibition of MLF. Because of this, MLF inhibition by bound SO_2 in the present study was caused by acetaldehyde bound SO_2, a finding in agreement with Fornachon (1963). In contrast, Larsen *et al.* (2003) and Hood (1983) suggested forms of bound SO_2 other than acetaldehyde bound SO_2 were responsible for MLF inhibition although no alternative forms were identified. Larsen *et al.* (2003) reported that V1116 inhibited MLF but because of the relative amounts of SO_2 and acetaldehyde measured, only a third of the SO_2 was bound to acetaldehyde. The remaining SO_2 was bound to compounds other than acetaldehyde. However, compared to Larsen *et al.* (2003), lower concentration of total SO_2 and higher concentrations of acetaldehyde were produced by yeast strains during this study and may account for these conflicting results. For example, Larsen *et al.* (2003) reported V1116 produced a maximum of 75 mg/L SO_2 during fermentation in a Chardonnay juice while we observed a maximum of only 46 mg/L of SO_2 being produced by V1116 in a Pinot gris juice. Larsen also reported a maximum acetaldehyde concentration produced by V1116 of approximately 18 mg/L versus 74.9 mg/L reported in this study. These discrepancies suggest that the form of bound SO_2 may be less important than the overall concentration of bound SO_2. Additional research should focus on whether strains of *O. oeni* vary in their susceptibility to bound SO_2 as well as its inhibitory action against other wine lactic acid bacteria, including spoilage organisms such as *Lactobacillus* and *Pediococcus*.

CONCLUSIONS

Yeast strains produced a range of SO_2 and SO_2 binding compounds during alcoholic fermentation in both a synthetic grape juice and a Pinot gris juice. Yeast that produced the highest levels of SO_2 most strongly inhibited the MLF. Little to no free SO_2 was measured during the fermentations, meaning that inhibition caused by SO_2 was likely due to bound SO_2. The form of bound SO_2 was predominately acetaldehyde bound SO_2 due to the high concentrations of acetaldehyde produced by the yeast. A change in the ratio of acetaldehyde, pyruvic acid and α-ketoglutarate bound SO_2 did not relieve the inhibition. If MLF is to be conducted then the concentration of bound SO_2, as well as free SO_2, needs to be considered while the form of bound SO_2 is less important. Furthermore, given the wide range of production of SO_2 binding compounds by yeast strains in this study the choice of yeast may significantly impact the SO_2 binding power of a wine and subsequently the amount of SO_2 needed to be added to maintain a target free SO_2 level.

LITERATURE CITED

Amerine, M.A. & Ough, C.S., 1964. Studies with controlled fermentation. VIII. Factors affecting aldehyde accumulation. Am. J. Enol. Vitic. 15, 23-33.

Avram, D. & Bakalinsky, A.T., 1997. *SSU1* encodes a plasma membrane protein with a central role in a network of proteins conferring sulfite tolerance in *Saccharomyces cerevisiae*. J. Bacteriol. 179, 5971-5974.

Azevedo, L.C., Reis, M.M., Motta, L.F., da Rocha, G.O., Silva, L.A. & Andrade, J.B., 2007. Evaluation of the formation of stability of hydroxyalkylsulfonic acids in wines. J. Agric. Food Chem. 55 (21), 8670-8680.

Barbe, J.C., de Revel, G., Joyeux, A., Lonvaud-Funel, A. & Bertrand, A., 2000. Role of carbonyl compounds in SO_2 binding phenomena in musts and wines from botrytized grapes. J. Agric. Food Chem. 48, 3413-3419.

Beelman, R.B., Keen, R.M., Banner, M.J. & King, S.W., 1982. Interactions between wine yeast and malolactic bacteria under wine conditions. Dev. Ind. Microbiol. 23, 107-121.

Britz, T.J. & Tracey, R.P., 1990. The combination effect of pH, SO_2, ethanol, and temperatures on the growth of *Leuconostoc oenos*. J. Appl. Bacteriol. 63, 23-31.

Buechsenstein, J.W. & Ough, C.S., 1978. SO_2 determination by aerations-oxidation: a comparison with Ripper. Am. J. Enol. Vitic. 29, 161-164.

Burroughs, L. F. & Sparks, A.H., 1973a. Sulphite binding power of wines and ciders. III Determination of carbonyl compound in a wine and calculation of its sulphite binding power. J. Sci. Food Agric. 24, 207-217.

Burroughs, L. F. & Sparks, A.H., 1973b. Sulphite binding power of wines and ciders. I Equilibrium constants for the dissociation of carbonyl-bisulfite compounds. J. Sci. Food Agric. 24, 187-198.

Capucho, I. & San Romao M.V., 1994. Effect of ethanol and fatty acids on malolactic activity of *Leuconostoc oenos*. Appl. Microbiol. Biotech. 42, 391-395.

Carr, J.G., Davies, P.A. & Sparks, A.H., 1976. The toxicity of sulphur dioxide towards certain lactic acid bacteria from fermented apple juice. J. Appl. Bacteriol. 40, 201-212.

Carrette, R., Teresa, M., Bordonsa, A. & Consantia, M., 2002. Inhibitory effect of sulfur dioxide and other stress compounds in wine on the ATPase activity of Oenococcus oeni. FEMS Microbiol. 211, 155-159.

Costello, P.J., Morrison, G.J., Lee, T.H. & Fleet, G.H., 1983. Numbers and species of lactic acid bacteria in wines during vinification. Food. Tech. Aust. 35, 14-18.

Davis, C.R., Wibowo, D., Eschenbruch, R., Lee, T.H. & Fleet, G.H., 1985. Practical implications of malolactic fermentation: A review. Am. J. Enol. Vitic. 34, 290-301.

Dick, K.J., Molan, P.C. & Eschenbruch, R., 1992. The isolation from *Saccharomyces cerevisiae* of two antibacterial cationic proteins that inhibit malolactic bacteria. Vitis, 31, 577-588.

Donalies, U.E.B. & Stahl, U., 2002. Increasing sulphite formation in *Saccharomyces cerevisiae* by over expression of MET14 and SSU1. Yeast, 19, 475-484.

Du Toit, M. & Pretorius, I.S., 2000. Microbial spoilage and preservation of wine: using weapons from nature's own arsenal - a review. South. Afr. J. Enol. Vitic. 21, 74-96.

Dukes, B.C. & Butzke, C., 1998. Rapid determination of primary amino acids in grape juice using an *o*-phthaldialdehyde/N-acetyl-L-cysteine spectrophotometric assay. Am. J. Enol. Vitic. 49, 125-134.

Edwards, C.G. & Beelman, R.B., 1987. Inhibition of the malolactic bacterium *Leuconostoc oenos* (PSU-1) by decanoic acid and subsequent removal of the inhibition by yeast ghosts. Am. J. Enol. Vitic. 38, 239-242.

Eglinton, J.M. & Henschke, P.A., 1996. *Saccharomyces cerevisiae* strains AWRI 838, Lavin EC1118 and Maurivin PDM do not produce excessive sulfur dioxide in white wine fermentations. Aust. J. Grape Wine Res. 2, 77-83.

Eschenbruch, R. (1974). Sulfite and sulfide formation during wine making. A review. Am. J. Enol. Vitic. 25, 299-302.

Fleet, G.H., Lafon-Lafourcade, S. & Ribéreau-Gayon, P., 1984. Evolution of yeasts and lactic acid bacteria during fermentation and storage of Bordeaux wines. Appl. Environ. Microbiol. 48, 1034-1038.

Fornachon, J.C.M., 1963. Inhibition of certain lactic acid bacteria by free and bound sulphur dioxide. J. Sci. Food Agric. 14, 857-862.

Fornachon, J.C.M., 1968. Influence of different yeasts on the growth of lactic acid bacteria in wine. J. Sci. Food Agric. 19, 374-378.

Fugelsang, K.C. & Edwards, C.G., 2007 (2nd ed). Wine microbiology: practical applications and procedures. Springer-Verlag, New York.

Hammond, S.M. & Carr, J.G., 1976. The antimicrobial activity of SO_2-with particular reference to fermented and non-fermented fruit juices. In: Skinner F.A. & Hugo, W.B. (eds). Inhibition and Inactivation of Vegetative Microbes. Academic Press, London. pp. 89-110.

Henick-Kling, T. & Park, Y.H., 1994. Considerations for the use of yeast and bacterial starter cultures: SO_2 and timing of inoculation. Am. J. Enol. Vitic. 45, 464-469.

Henick-Kling, T., Edinger, W., Daniel, P. & Monk, P., 1998. Selective effects of sulfur dioxide and yeast starter culture addition on indigenous yeast populations and sensory characteristics of wine. J. Appl. Microbiol. 84, 865–876.

Hinze, H. & Holzer, H., 1986. Analysis of the energy metabolism after incubation of *Saccharomyces cerevisiae* with sulfite or nitrite. Arch. Microbiol. 145, 27-31.

Hood, A., 1983. Inhibition of growth of wine lactic-acid bacteria by acetaldehyde-bound sulphur dioxide. Aust. N.Z. Grape Grow. Winemaker, 232, 34-43.

Jarvis, B. & Lea, A.G.H., 2000. Sulphite binding in ciders. Int. J. Food Sci. Tech. 35, 113-127.

King, S.W. & Beelman, R.B., 1986. Metabolic interactions between *Saccharomyces cerevisiae* and *Leuconostoc oenos* in a model grape juice/ wine system. Am. J. Enol. Vitic. 37.53-60.

Kunkee, R.E., 1967. Malo-lactic fermentation. Adv. Appl. Microbiol. 9, 235-279.

Kunkee, R.E., Pilone, G.J. & Combs, R.E., 1965. The occurrence of malo-lactic fermentation in southern California wines. Am. J. Enol. Vitic. 16, 219-223.

Lafon-Lafourcade, S., Carre, E. & Ribéreau-Gayon, P., 1983. Occurrence of lactic acid bacteria during the different stages of vinification and conservation of wines. Appl. Environ. Microbiol. 46, 874-880.

Larsen, J.T., Nielsen, J.C., Kramp, B. Richelieu, M., Riisager, M.J., Arnebord, N. & Edwards, C.G., 2003. Impact of different strains of *Saccharomyces cerevisiae* on malolactic fermentation by *Oenococcus oeni*. Am. J. Enol. Vitic. 54, 246-251.

Lea, A.G.H., Ford, G.D. & Fowler, S., 2000. Analytical techniques for the estimation of sulfite binding components in ciders and wines. Int. J. Food Sci. Tech. 35, 105-112.

Lonvaud-Funel, A., Joyeuxm, A. & Dessens, C., 1988. Inhibition of malolactic fermentation of wines by products of yeast metabolisms. J. Sci. Food Agric. 44, 183-191.

Margalith, P.Z., 1981. Flavor Microbiology. Charles C. Thomas Publishers, Springfield, Illinois.

Martinez, P., Rodriguez, L. & Benitez, T., 1997. Evolution of flor yeast production during the biological aging of fino sherry wine. Am. J. Enol. Vitic. 48, 160-168.

Mendoza, L.M., Manca de Nadra, M.C. & Farias, M.E., 2010. Antagonistic interaction between yeasts and lactic acid bacteria of oenological relevance: partial characterization of inhibitory compounds produced by yeasts. Food Res. Int. 43, 1990-1998.

Nehme, N., Mathieu, F. & Taillandier, P., 2010. Impact of the co-culture of *Saccharomyces cerevisiae-Oenococcus oeni* on malolactic fermentation and partial characterization of a yeast-derived inhibitory peptidic fraction. Food. Microbiol. 27, 150-157.

Osborne, J. P. & Edwards, C.G., 2006. Inhibition of malolactic fermentation by *Saccharomyces* during alcoholic fermentation under low and high nitrogen conditions. Aust. J. Grape Wine Res. 12, 69-78.

Osborne, J.P. & Edwards, C.G., 2007. Inhibition of malolactic fermentation by a peptide produced by *Saccharomyces cerevisiae* during alcoholic fermentation. Int. J. Food Microbiol. 118, 27-34.

Ough, C.S., 1985. Effects of temperature and SO_2 on wine during simulated transport or storage. Am. J. Enol. Vitic. 36, 18-22.

Paynowski, R.J., Jiranek, V. & Markides, A.J., 2002. Yeast viability during fermentation and *sur lie* aging of a defined medium and subsequent growth of *Oenococcus oeni*. Aust. J. Grape Wine Res. 8, 62-69.

Peynaud, E., Blouin, L. & Lafon-Lafourcade, S., 1966. Review of applications of enzymatic methods to the determination of some organic acids in wines. Am. J. Enol. Vitic. 17, 218-224.

Pilkington, B.J. & Rose, A., 1988. Reactions of *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii* to sulphite. J. Gen. Microbiol. 134, 2823-2830.

Rankine, B.C., 1965. Factors influencing the pyruvic acid content of wines. J. Sci. Food Agric. 16, 394-398.

Rankine, B.C., 1967. Influence of yeast strain and pH on pyruvic acid content of wines. J. Sci. Food Agric. 18, 41-44.

Rankine, B.C., 1968. Formation of α-ketoglutaric acid by wine yeasts and it's oenological significance. J. Sci. Food Agric. 19, 624-627.

Rankine, B.C. & Pocock, K.F., 1969. Influence of yeast strain on binding of SO_2 in wines on its formation during fermentation. J. Sci. Food Agric. 20, 22-49.

Ribéreau-Gayon, J., Peynaud, E. & Lafon, M., 1956. Investigations on the origin of secondary products of alcoholic fermentation. Am. J. Enol. Vitic. 7, 53-61.

Romano, P., Suzzi, G., Turbanit, L. & Polsinelly, M., 1994. Acetaldehyde production in *Saccharomyces cerevisiae* wine yeast. FEMS Lett. 118, 213-218.

Rose, A.H. & Pilkington, B.J., 1989. Sulfite. In: Gould, G.W. (ed). Mechanisms of Action of Food Preservation. Elsevier Applied Science, London. pp. 201-223.

Stratford, M., Morgan, P. & Rose, A.H., 1987. Sulphur dioxide resistance in *Saccharomyces cerevisiae* and *Saccharomyces ludwigii*. J. Gen Microbiol. 133, 2173-2179.

Thomas, D. & Surdin-Kerjan, Y., 1997. Metabolism of sulfur amino acids in *Saccharomyces cerevisiae*. Microbiol. Mol. Biol. Rev. 61(4), 503-532.

Van Vuuren, H.J.J. & Dicks, L.M.T., 1993. *Leuconostoc oenos*: a review. Am. J. Enol. Vitic. 44, 99-112.

Versari, A., Parpinello, G.P. & Cattaneo, M., 1999. *Leuconostoc oenos* and malolactic fermentation in wine; a review. J. Ind. Microbiol. Biotech. 23, 447-455.

Wang, X.D., Bohlscheid, J.C. & Edwards, C.G., 2003. Fermentative activity and production of volatile compounds by *Saccharomyces* grown in synthetic grape juice media deficient in assimilable nitrogen and/or pantothenic acid. J. Appl. Microbiol. 94, 349-359.

Weeks, C., 1969. Production of sulfur dioxide-binding compounds and of sulfur dioxide by two *Saccharomyces* yeast. Am. J. Enol. Vitic. 20, 32-39.

Wibowo, D., Eschenbruch, R.E., Davis, C.R., Fleet, G.H. & Lee, T.H., 1985. Occurrence and growth of lactic acid bacteria in wine: A review. Am. J. Enol. Vitic. 36, 302-313.

Wibowo, D., Fleet, G.H., Lee, T.H. & Eschenbruch, R.E., 1988. Factors affecting the induction of malolactic fermentation in red wines with *Leuconostoc oenos*. J. Appl. Bacteriol. 64, 421-428.

Wurdig, G., 1989. Behandlung des weines mit schwefliger saure. Da: Wurdig, G. & Woller, H. (eds). Chemie des Weines. Ulmer Verlag, Stuttgart.

Zoecklein, B.W., Fugelsang, K.C., Gump, B.H. & Nury, F S., 1995. Wine Analysis and Production. Chapman and Hall, New York.